Selective Cytotoxic Activity of Grape Peel and Seed Extracts Against Oral Tumor Cell Lines

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Abstract. *Grape seed extracts were more cytotoxic than grape peel extracts. Methanol and 70% methanol extracts of grape seed selectively killed two human oral tumor cell lines, more efficiently than human gingival fibroblasts. ESR spectroscopy revealed that these extracts produced radicals under alkaline conditions and enhanced the radical intensity of sodium ascorbate at higher concentrations. On the other hand, lower concentration of these extracts slightly reduced the radical intensity of sodium ascorbate, and scavenged superoxide anion, generated by hypoxanthine and xanthine oxidase reaction. These properties of grape seed extracts suggest their possible application for cancer prevention.*

There is a French paradox that red wine drinkers with a high fat diet in Europe have low risk for coronary atherosclerosis (1). Seed of grape contains dietary fibers rich in hemicelluloses (2, 3). Red wine, which contains a lot of polyphenols, inhibits the copper-catalysed oxidation of normal human low-density lipoprotein (LDL), more efficiently than α-tocopherol (1). This suggests that the polyphenols can act as an antioxidant which inhibits LDL oxidation (1). Oxidation of polysaturated lipid components of LDL by active oxygens might produce coronary atherosclerosis (4, 5). Ascorbate and α-tocopherol inhibits copper-induced *in vitro* oxidation (6). Flavonoids also inhibit the *in vitro* oxidation of human LDL (7, 8, 9). High and moderate plasma concentrations of ascorbic acid and α-tocopherol might prevent coronary heart disease (CHD) (10). Natural flavonoids can donate hydrogen (H) or react with superoxide anions (11), hydroxyl radicals (12), and lipid peroxyl radicals (13). We have recently reported that ascorbates (14), gallates (15), benz[*c*]acridines (16), benzo[*a*]phenothiazines (16), dopamine (17) or *N*-acylphenothiazines derivatives (18) produce radicals, and induce apoptosis or differentiation in various tumor cell lines. We investigated here whether grape peel and seed extracts kill human oral tumor cell lines (HSC-2, HSG) more efficiently than human gingival fibroblasts HGF.

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Materials and Methods

Materials. The popular Japanese grape, *Vitis vinifera* "Koshu" (Vitaceae), was supplied by Sapporo Wine Katsunuma Winery, Yamanashi Prefecture, Japan. The peels and seeds of grapes were manually separated. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle minimum essential medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); diethylenetriaminepentaacetic acid (DETAPAC) (Sigma Chem. Co., St. Louis, MO, USA).

Preparation of grape extracts. Peels of "Koshu" grapes (1.1 kg) were successively extracted with hexane, acetone, MeOH and 70% MeOH at room temperature and the solvent was evaporated *in vacuo* to obtain the hexane extract [**GPP-1 H0**] (27.7 g), acetone extract [**GPP-1 A0**] (71.2 g), MeOH extract [**GPP-1 M0**] (61.0 g) and 70% MeOH extract [**GPP-1 70M0**] (25.0 g), respectively (Figure 1).

Similary, seeds of "Koshu" grapes (570 g) were successively extracted with hexane, acetone, MeOH and 70% MeOH at room temperature and the solvent was evaporated *in vacuo* to obtain the hexane extract [**GSP-1 H0**] (50.2 g), acetone extract [**GSP-1 A0**] (23.5 g), MeOH extract [**GSP-1 M0**] (51.0 g) and 70% MeOH extract [**GSP-1 70M0**] (13.0 g), respectively (Figure 1).

Assay for cytotoxic activity. Human oral squamous cell carcinoma cells (HSC-2), human oral salivary grand tumor cells (HSG) and human oral gingival fibroblasts (HGF) (5-7 population doubling levels) were cultured in DMEM supplemented with 10% heat-inactivated FBS. These cells were incubated for 24 hours with the indicated concentrations of test samples, and the viable cell number was then determined by MTT method. In brief, the cells were washed with phosphate-buffered saline (PBS), and incubated for 4 hours with fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, cells were lysed with 100

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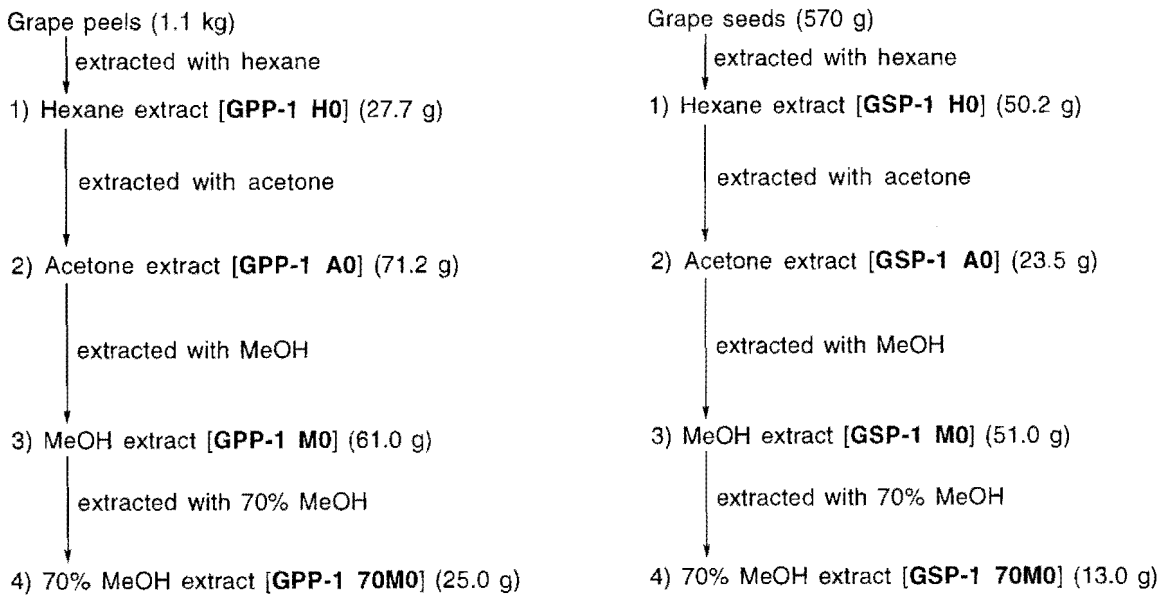

Figure 1. *Fractional procedures of grape peel and seed extracts.*

μL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate with Labsystems Multiskan^{R} (Biochromatic) with Star/DOT Matrix printer JL-10. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Assay for radical intensity. Radical intensity of four GSP-1 extracts was determined at 25°C using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 335.6 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 500; time constant, 0.1 sec; scanning time, 4 min. Radical intensity was determined in 0.1 M Tris-HCl (pH 7.4, 8.0), 0.1M $NaHCO_3/Na_2CO_3$ (pH 9.0, 10.0), or 0.1N KOH (pH12.5)-50% DMSO and the radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (14, 15, 19, 20).

Radical scavenging activity against superoxide anion (O_2^-). O_2^- was generated by hypoxanthine (HX) and xanthine oxidase (XOD) reaction (200 μL) [2 mM (in PBS) HX 50 μL, 0.5 mM DETAPAC 20 μL, 5% DMPO/PBS 50 μL, sample 50 μL in 60% DMSO, XOD (0.5 U/mL) 30 μL]. The time constant and scanning time was changed to 0.03 sec and 1 min, respectively.

Results and Discussion

Cytotoxic activity. Cytotoxic activity of grape peel and seed extracts against two human oral tumor cell lines (HSC-2, HSG) and human gingival fibroblasts (HGF) was investigated. All four fractions of grape peel extracts (**GPP-1**): hexane, acetone, MeOH and 70% MeOH extracts ([**GPP-1 H0**], [**GPP-1 A0**], [**GPP-1 M0**], [**GPP-1 70M0**]) showed very weak cytotoxicity against all three cell lines (Table I). Two fractions of grape seed extracts, [**GSP-1 M0**] and [**GSP-1 70M0**]), were relatively cytotoxic to HSC-2 cells (CC_{50}=146 and 154 μg/mL, respectively), as compared with HSG (CC_{50}=221-243 μg/mL) and HGF (CC_{50}>500 μg/mL) (Table I).

Table I. *Cytotoxic activity of grape peel (**GPP-1**) and seed extracts (**GSP-1**) against tumor and normal cells.*

Fraction	Cytotoxic activity (CC_{50}: μg/mL)		
	Human oral tumor cell line		Human gingival fibroblast
	HSC-2	HSG	HGF
GPP-1 H0	>500	>500	>500
GPP-1 A0	>500	>500	>500
GPP-1 M0	>500	>500	>500
GPP-1 70M0	492	>500	>500
GSP-1 H0	490	>500	>500
GSP-1 A0	>500	>500	>500
GSP-1 M0	146	221	>500
GSP-1 70M0	154	243	>500

Radical generation. All four fractions of grape seed extracts ([**GSP-1 H0**], [**GSP-1 A0**], [**GSP-1 M0**], [**GSP-1 70M0**]) produced no detectable ESR signal of radicals below pH 9.0. However, three extracts of them ([**GSP-1 A0**], [**GSP-1 M0**],

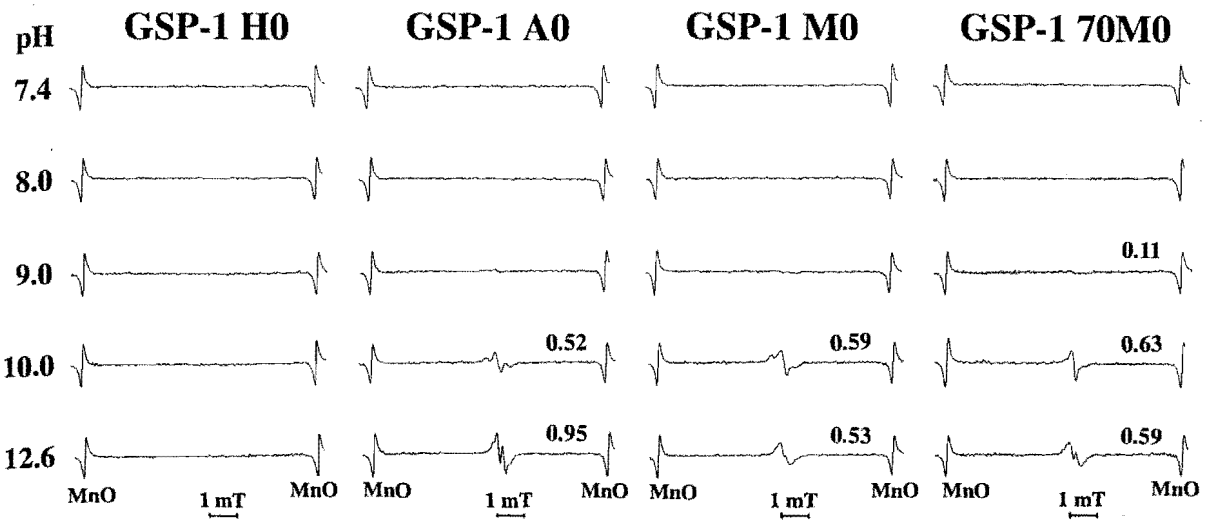

Figure 2. *ESR spectra of four fractions of grape seed extracts ([GSP-1 H0], [GSP-1 A0], [GSP-1 M0] and [GSP-1 70M0]) (3 mg/mL) in each buffer solution (at the indicated pH) containing 50% DMSO. The gain and scanning time were changed to 630 and 2 min, respectively.*

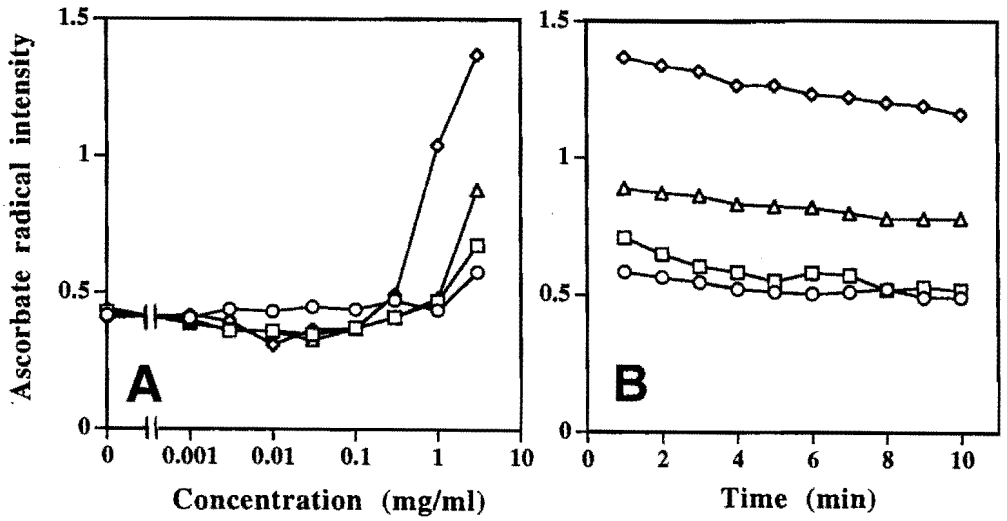

Figure 3. *Effect of GSP-1 extracts on the radical intensity of sodium ascorbate (3 mM) (A) and the stability of ascorbate radical (B) in 0.08M Tris-HCl, pH 8.0.* ○*: GSP-1 H0;* □*: GSP-1 A0;* △*: GSP-1 M0;* ◇*: GSP-1 70M0.*

[**GSP-1 70M0**]) produced radicals at higher pH (Figure 2). The relative radical intensity of these fractions at pH10.0 was in the order of [**GSP-1 H0**](0)<[**GSP-1 A0**](0.52)<[**GSP-1 M0**](0.59)<[**GSP-1 70M0**](0.63). At pH 12.6, their radical intensity reached a plateau level (Figure 2).

[**GSP-1 70M0**] slightly reduced the radical intensity of sodium ascorbate at lower concentration (<0.01 mg/mL), but enhanced the radical intensity at higher concentrations (>0.1 mg/mL) (Figure 3A). The radical enhancing effect of [**GSP-1 M0**] was slightly lower, whereas that of [**GSP-1 A0**] and [**GSP-1 H0**] was much less. The ascorbate radicals in the presence of any of these extracts were stable for 10 min (Figure 3(B)). Polyphenols in the **GSP-1** extracts are good electron donors, which is identified by their reduction of the oxoferryl radical of myoglobin, generated by the interaction of equimolar amounts of myoglobin and hydrogen peroxide (21), or flavonoids, in the presence of ascorbic acid, could convert the Cu^{+} complex to the Cu^{2+} complex by electron reduction,

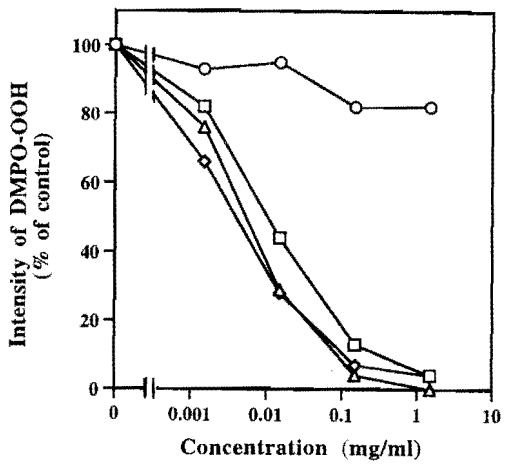

Figure 4. *O_2^- scavenging activity of four fractions of GSP-1 extracts. The intensity of O_2^- generated by HX-XOD reaction in the presence of the indicated concentrations of ○: GSP-1 H0; □: GSP-1 A0; △: GSP-1 M0; ◇: GSP-1 70M0, was defined as the ratio of peak height of MnO. DMSO was present at the final concentration of 15% in all samples.*

reducing hydroperoxides (22). Flavonoids inhibit human low-density lipoproteins (LDL) oxidation, thus reducing the incidence of atherosclerosis (8).

Radical scavenging activity against superoxide anion (O_2^-). All four fractions of **GSP-1** extracts scavenged O_2^- to various extents. **[GSP-1 70M0]** showed the greatest O_2^- scavenging activity, followed by **[GSP-1 M0], [GSP-1 A0]** and **[GSP-1 H0]** (Figure 4). These O_2^- scavenging activity of grape seed extracts suggests their possible application for cancer prevention.

The present study demonstrates that **GSP-1** extracts showed the selective cytotoxicity against human oral tumor cell lines (HSC-2, HSG), as compared with human gingival fibroblasts HGF, and their activity relates to their radical production or prooxidant action. Further studies with a number of tumor and normal cell lines are neccessary to confirm the tumor specificity of these extracts.

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